DEVELOPMENT, CHARACTERIZATION AND VALIDATION OF ANTI-HUMAN H_3 RECEPTOR ISOFORM SPECIFIC ANTIBODIES

FC Shenton*, N Lethbridge* and PL Chazot#

School of Biological & Biomedical Sciences, Durham University, South Road, Durham, DH1 3LE

** Equal contribution to this book chapter*

#Corresponding author

**Equal contribution from first two authors*

Abstract

Generation of selective anti-GPCR antibodies is a challenging pursuit, and the anti-histamine receptor antibodies are no exception. Furthermore, producing isoform-selective antibodies is even more of a challenge. The first anti-histamine H_3 receptor (H_3) antibodies were developed in 2001 and validated in 2007. The first two antibodies were raised against human H_3 receptor sequences common to most human and rodent isoforms. This chapter describes how a panel of anti-hH_3 receptor isoform specific antibodies have been generated, characterised and validated for selectivity (individual rH_3R and hH_3R isoform transient expression in HEK293 cells, isoform peptide blockade and the use of H_3R KO mice), and utility for a range of immunobiochemical techniques (immunoblotting, immunohistochemical and immunofluorescence techniques).

Introduction

Due to lower than expected homology with histamine receptors H_1 and H_2, the H_3 receptor was not cloned until 1999 **(1)** more than a decade after it was first described by seminal studies in France **(2)** It was discovered as part of an effort to identify orphan GPCRs. Lovenberg and colleagues identified a partial clone (GPCR97) that had significant homology to a range of biogenic amine receptors. The GPCR97 clone was used to probe a human thalamus library, which resulted in the isolation of a full-length clone encoding a putative G protein- coupled receptor. Homology analysis showed the highest similarity to M_2 muscarinic acetylcholine receptors and overall low homology to all other biogenic amine receptors. Subsequent analysis revealed a pharmacological profile practically indistinguishable from that for the histamine H_3 receptor. In two analyses of the human gene **(3,4)** a 1063 base pair (bp) intron beginning 250 nucleotides downstream of the start codon (corresponding to aa 84) and another 1564 bp intron at position 417 (corresponding to aa 139) suggests a gene with three exons and two introns. By contrast Cogé **(5)** has put forward a gene having a minimum of

four exons and three introns. In their analysis the coding region is interrupted by introns of 1062, 1565 and 240 bp. A further exon may result in an additional 8 amino acids at the C-terminus, leading to a protein of 453 aa. The result of these diverse exon/intron junctions is the possibility of alternative splicing to generate multiple splice variants which may potentially lead to several receptor isoforms.

The cloning of the H_3 receptor initiated a new era in histamine research. The existence of multiple H_3 receptor isoforms could contribute to the pharmacological heterogeneity in H_3 receptors, within and across species, which has long been recognised, but not understood. In all species tested so far the full length H_3 receptor encodes a polypeptide of 445 amino acids (predicted polypeptide Mr 47,000). Shorter isoforms with deletions predominantly in the third intracellular loop domain have also been identified (predicted polypeptide M_r range 20,000-45,000). While the full length clone is found in most abundance in the CNS in all species studied so far, there is regional variation in the distribution of the mRNAs encoding the different isoforms **(5)**; this has given rise to speculation that H_3 heterogeneity could underlie different activities and functions of H_3 receptors in specific tissues **(6)**.

The evidence for heterogeneity in anatomical distribution was based initially on the distribution pattern of mRNA coding for the various isoforms. Note that these splicing events yield potentially different protein sequences in rodents and human H_3R orthologs. In order to define the importance of human and rodent H_3 receptor heterogeneity, specific immunological probes are required.

Generation of selective anti-GPCR antibodies is a challenging pursuit, and the histamine receptors are no exception. Furthermore, producing isoform-selective antibodies is even more testing. The first anti-histamine H_3 receptor (H_3) antibodies were developed in 2001 and validated using KO Mice in 2007 **(7,8)**. The first two antibodies were raised against human H_3 receptor sequences common to most human and rodent isoforms: anti-hH_3 (346-358) and anti-hH_3 (175-187), respectively. They both detected two specific immunoreactive protein species (Mr 68,000 and 93,000) which were suppressed by prior incubation with their respective peptide antigens, in many adult rat and mouse brain regions. These protein species are most likely derived from dimeric and/or glycosylation variants of the receptor.

In an attempt to generate an antibody specific to the rat H_{3C} (397) isoform a peptide sequence was chosen which spanned the deletion within the third intracellular loop rat isoform sequences, and should therefore be unique to this isoform (Figure 1). However, when tested against three of the major human and rat isoforms heterologously

expressed in HEK 293 cells namely $hH_{3\ (445),}$ $hH_{3\ (365)}$ and $hH_{3\ (329)}$; $rH3_{A\ (445)}$, $rH3_{B\ (413)}$ and $rH3_{C\ (397)}$, the antibody was found to be selective for both the full length human and rat isoforms ($hH_{3\ (445)}$ and $rH_{3A\ (445)}$) as well as the expected $rH_{3C\ (397)}$ isoform. Using this same strategy antibodies specific for three of the other major human H_3R isoforms: 415, 365, and 329 ΔI3 (see Fig 2 human isoform sequences), were also sought. Finally a peptide sequence unique to the heavily truncated human $H_{3\ 220}$ was selected (**(9)**, Isoform 5). Human $H_{3\ (220)}$ has a 170-306 deletion plus a frame shift and a novel stop codon, so that the C terminus is unique to this isoform. Therefore, the last 10 amino acids of human $H_{3\ 220}$ were selected as immunogen.

Herein, methods are described for rationale selection and preparation of the peptides for immunization, the immunization procedure, antibody purification and the experiments performed to validate the individual antibody specificities. Results of immunohistochemical analysis using the human/rodent antibody in mouse brain slices from both wildtype (WT) and H_3R -/- (H_3R KO) mice (kind gift of Prof Tim Lovenberg, Johnson & Johnson) are also presented as exemplars.

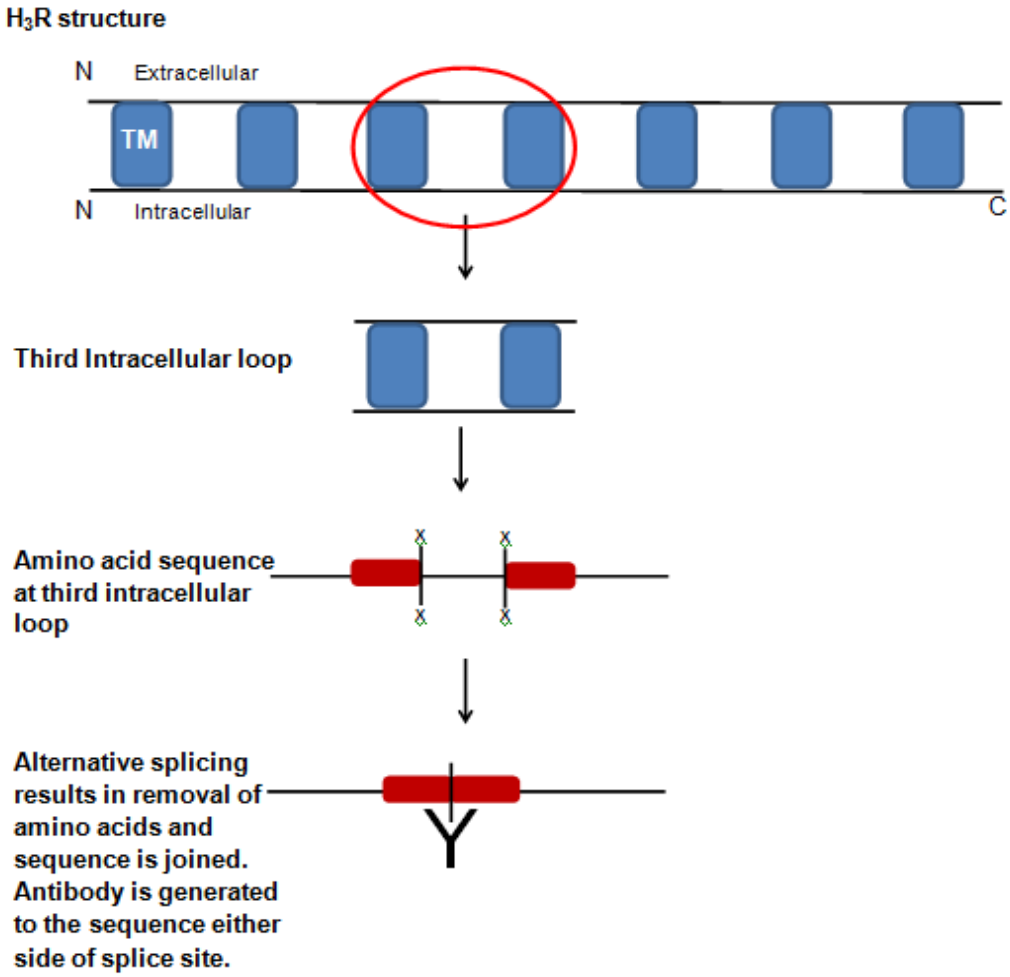


Figure 1 *Schematic showing how peptide sequences were selected in an attempt to produce isoform specific antibodies against the distinctly spliced third intracellular loop of the human H_3R. Amino acid sequences were chosen that spanned either side of the splicing event so once the sequence was removed and the sequence at either side joined, the antibody generated would hopefully detect the sequence. The sequence selected was long enough so that the antibody was specific but not too long that the antibody would detect other closely related isoforms.*

Herein, the methods for preparing peptides for immunisation, the immunisation procedure, antibody purification and the experiments performed to check the individual antibody specificity are described.

Peptide sequences chosen for each isoform:

(A) Full length human H_3R sequence.

Peptide sequence used to generate anti-pan H_3R antibody, blue

1 merappdgpl nasgalagea aaaggargfs aawtavlaal mallivatvl gnalvmlafv

61 adsslrtqnn ffllnlaisd flvgafcipl yvpyvltgrw tfgrglcklw lvvdyllcts

121 safnivlisy drflsvtrav syraqqgdtr ravrkmllvw vlafllygpa ilsweylsgg
181 ssipeghcya effynwyfli tastlefftp flsvtffnls iylniqrrtr lrldgareaa
241 gpepppeaqp sppppgcwg cwqkghgeam plhrygvgea avgaeageat lgggggggsv
301 asptsssgss srgterprsl krgskpsass aslekrmkmv sqsftqrfrlsrdrkvaksl
361 avivsifglc wapytllmii raachghcvp dywyetsfwl lwansavnpv lyplchhsfr
421 raftkllcpq klkiqphssl ehcwk

(B) Full length human H_3R_{445} sequence.

Peptide sequence used to generate anti-r$H_3R_{A/C}$ and h$H_3R_{453/445}$antibody, blue

1 merappdgpl nasgalagea aaaggargfs aawtavlaal mallivatvl gnalvmlafv
61 adsslrtqnn ffllnlaisd flvgafcipl yvpyvltgrw tfgrglcklw lvvdyllcts
121 safnivlisy drflsvtrav syraqqgdtr ravrkmllvw vlafllygpa ilsweylsgg
181 ssipeghcya effynwyfli tastlefftp flsvtffnls iylniqrrtr lrldgareaa
241 gpepppeaqp sppppgcwg cwqkghgeamplhrygvgea avgaeageat lgggggggsv
301 asptsssgss srgterprsl krgskpsass aslekrmkmv sqsftqrfrl srdrkvaksl
361 avivsifglc wapytllmii raachghcvp dywyetsfwl lwansavnpv lyplchhsfr
421 raftkllcpq klkiqphssl ehcwk

(C) Human sequence showing the H_3R_{453} isoform *(***Addition of 8 amino acids in green)**.

Peptide sequence used to generate: anti-human $H_3R_{445/453}$ antibody, blue

1 merappdgpl nasgalagea aaaggargfs aawtavlaal mallivatvl gnalvmlafv
61 adsslrtqnn ffllnlaisd flvgafcipl yvpyvltgrw tfgrglcklw lvvdyllcts
121 safnivlisy drflsvtrav syraqqgdtr ravrkmllvw vlafllygpa ilsweylsgg
181 ssipeghcya effynwyfli tastlefftp flsvtffnls iylniqrrtr lrldgareaa
241 gpepppeaqp sppppgcwg cwqkghgeamplhrygvgea avgaeageat lgggggggsv
301 asptsssgss srgterprsl krgskpsass aslekrmkmv sqsftqrfrl srdrkvaksl
361 avivsifglc wapytllmii raachghcvp dywyetsfwl lwansavnpv lyplchhsfr
421 raftkllcpq klkiqphssl ehcwk**rrprprwrsa**

(D) Human sequence showing the H_3R_{365} deletion / isoform 2 **(deletion in red and bold)**.

Peptide sequence used to generate: anti-human H_3R_{365} peptide 1 antibody, blue

1 merappdgpl nasgalagea aaaggargfs aawtavlaal mallivatvl gnalvmlafv
61 adsslrtqnn ffllnlaisd flvgafcipl yvpyvltgrw tfgrglcklw lvvdyllcts
121 safnivlisy drflsvtrav syraqqgdtr ravrkmllvw vlafllygpa ilsweylsgg
181 ssipeghcya effynwyfli tastlefftp flsvtffnls iylniqrrtr lrldgareaa

241 gpepppeaqp sppppgcwg cwqkghgeam plhrygvgea avgaeageat lgggggggsv

301 asptsssgss srgterprsl krgskpsass aslekrmkmv sqsftqrfrl srdrkvaksl

361 avivsifglcwapytllmii raachghcvp dywyetsfwl lwansavnpv lyplchhsfr

421 raftkllcpq klkiqphssl ehcwk

(E) Human sequence showing the H_3R_{365} deletion / isoform 2 **(deletion in red and bold)**.

Peptide sequence used generate: anti-human H_3R_{365} peptide 2 antibody, blue

1 merappdgpl nasgalagea aaaggargfs aawtavlaal mallivatvl gnalvmlafv

61 adsslrtqnn ffllnlaisd flvgafcipl yvpyvltgrw tfgrglcklw lvvdyllcts

121 safnivlisy drflsvtrav syraqqgdtr ravrkmllvw vlafllygpa ilsweylsgg

181 ssipeghcya effynwyfli tastlefftp flsvtffnls iylniqrrtr lrldgareaa

241 gpepppeaqp sppppgcwg cwqkghgeam plhrygvgea avgaeageat lgggggggsv

301 asptsssgss srgterprsl krgskpsass aslekrmkmv sqsftqrfrl srdrkvaksl

361 avivsifglcwapytllmii raachghcvp dywyetsfwl lwansavnpv lyplchhsfr

421 raftkllcpq klkiqphssl ehcwk

(F) Human sequence showing the H_3R_{329} deletion / isoform 2 **(deletion in red and bold)**. Peptide generated to the sequence at position 222-231 (anti-hH_3R_{329} antibody).

Peptide sequence used to generate:anti-human H_3R_{329} antibody, blue

1 merappdgpl nasgalagea aaaggargfs aawtavlaal mallivatvl gnalvmlafv

61 adsslrtqnn ffllnlaisd flvgafcipl yvpyvltgrw tfgrglcklw lvvdyllcts

121 safnivlisy drflsvtrav syraqqgdtr ravrkmllvw vlafllygpa ilsweylsgg

181 ssipeghcya effynwyfli tastlefftp flsvtffnls iylniq**rrtr lrldgareaa**

241 **gpepppeaqp sppppgcwg cwqkghgeam plhrygvgea avgaeageat lgggggggsv**

301 asptsssgss srgterprsl krgskpsass aslekrmkmv sqsftqrfrl srdrkvaksl

361 avivsifglc wapytllmii raachghcvp dywyetsfwl lwansavnpv lyplchhsfr

421 raftkllcpq klkiqphssl ehcwk

(G) Human sequence showing the H_3R_{200} deletion / isoform 5 **(deletion in red and bold)**. (Peptide sequence used to generate: anti-human H_3R_{200} antibody, blue

1 merappdgpl nasgalagea aaaggargfs aawtavlaal mallivatvl gnalvmlafv

61 adsslrtqnn ffllnlaisd flvgafcipl yvpyvltgrw tfgrglcklw lvvdyllcts

121 safnivlisy drflsvtrav syraqqgdtr ravrkmllvw vlafllygpa**ilsweylsgg**

181 **ssipeghcya effynwyfli tastlefftp flsvtffnls iylniqrrtr lrldgareaa**

241 **gpepppeaqp sppppgcwg cwqkghgeam plhrygvgea avgaeageat lgggggggsv**

301 **asptsssgss srgterprsl krgskpsass aslekrmkmv sqsftqrfrl srdrkvaksl**

361 **avivsifglc wapytllmii raachghcvp dywyetsfwl lwansavnpv lyplchhsfr**

421 **raftkllcpq klkiqphssl ehcwk paaprgalrg rahsrgapsr** rrprprwrsa

Figure 2 *The amino acid sequences of the full length human H_3R and its isoforms and the peptide sequences used to generate specific antibodies to those isoforms. (A) H_3R amino acid coding sequence, (B) H_3R_{445} amino acid sequence and selected immunogen sequence. (C) H_3R_{365} amino acid sequence with the area spliced out highlighted in red and selected immunogen sequence in blue. (D) H_3R_{365} amino acid sequence with the area spliced out highlighted in red and selected immunogen sequence in blue. H_3R_{329} amino acid sequence with the area spliced out highlighted in red and selected immunogen sequence in blue. H_3R_{200} amino acid sequence with the area spliced out highlighted in red and sequence highlighted to raise an antibody against selected immunogen sequence in blue.*

Methods

Choice of Peptide Sequences

Specific peptide sequences were chosen to immunize rabbits to produce anti-peptide antibodies (Figure 2). The peptide was chosen based on the peptide specificity being unique to the particular isoform and its likely immunogenicity using the rationale described herein and previously **(10)**. A cysteine residue was added to one end of each sequence so that the peptide could be *directionally* coupled to a carrier protein, keyhole limpet hemocyanin (KLH) or thyroglobulin to ensure the appropriate configuration of peptide was maintained:

Figure 3 Anti-human H_3R isoform Antibody Panel

Ab 1 was generated against a sequence specific to the i3 region in the rat H_{3C} isoform

rH_{3C} (268 – 277) EAMPLHRGSK-Cys

Human H_3R isoform specific antibodies:

Ab 2 Human $H_3R_{(365)}$ peptide 1	(268 – 281)	EAMPLHRKVAKSLA-Cys
Ab 3 Human $H_3R_{(365)}$ peptide 2	(268 – 278)	Cys-EAMPLHRKVAK
Ab 4 Human $H_3R_{(329)\ \Delta I3}$	(222 – 231)	Cys-YLNIQSFTQR
Ab 5 human $H_3R_{(200)}$	(191 – 200)	Cys-RRPRPRWRSA

Antibody Production

The 10-15-mer peptide alone is too small to stimulate an adequate immune response and is therefore conjugated to a large immunogenic carrier protein before being injected into the rabbit (female Dutch and NZ white rabbits are commonly used breeds). The peptide is conjugated using Imject® Maleimide activated mcKLH kit to illicit an enhanced humoral immune response. The rabbit serum is collected and purified using peptide affinity chromatography.

The Imject Maleimide activated mcKLH method of coupling peptides to carrier proteins

The method was used to conjugate the peptide to the carrier protein KLH through its carboxyl-terminal cysteine residue. There are other coupling strategies available, including Amino- and Carboxy-terminal coupling described in other publications **(10).**

1. Dissove one vial of maleimide-activated mcKLH by adding 200 µl of distilled water, making a 10 mg/ml solution. 200 µl of conjugation buffer was added to 5 mg synthetic peptide (**Note 1**).
2. Imediately add the peptide solution to the reconstituted mcKLH. Incubate the carrier protein and peptide mixture for a further 2 hours at room temperature with gentle agitation.
3. Separate the conjugated peptide from EDTA by desalting. Dissolve one bottle of purification buffer salts in 60 ml of distilled water. Remove the top and bottom caps from a desalting column and wash 3 times with 15 ml of purification buffer salts. Add the hapten-carrier mixture to the centre of the column disc. Apply to the column and collect each of the fractions sequentially.
4. Read the absorbency of each fraction at 280 nm to detect the fractions containing the conjugate. Pool the peak fractions, and re-read the absorbence. Dilute the peptide protein conjugate with PBS to a final concentration of 1 mg/ml and store in 100 µl aliquots at -20^{o}C.

Note 1: Synthetic peptides can be purchased commercially from various companies (eg. Cambridge Research Biochemical, Billingham, UK), at >98% purity is sufficient. Ideally at least 15 mg are required for all procedures.

Inoculation Procedure

1. Carefully mix 200 µl of sterile PBS with 100 µg of freshly thawed peptide-carrier protein conjugate, and emulsify with an equal volume of Freund's adjuvant (Sigma, UK) using a 1 ml syringe (**Note 2**).
2. All procedures are performed in accordance with the UK Animals Scientific Procedures Act 1986, or equivalent.
3. Inject the Conjugate-Adjuvant preparation equally using a 25-gauge needle intramuscularly into both hind legs of a Dutch or New Zealand White rabbit (**Note 3**). Perform the primary immunisation using complete Freund's adjuvant, while for subsequent immunisations (at 1 month intervals) use Freund's incomplete adjuvant.
4. Bleed rabbits (**See Note 3**) from the marginal ear vein 7 – 10 days following the booster injections, and collect 10 – 15 ml of blood. Allow blood to stand at room temperature for 2 hours, and then for 16 hours at 4^{o}C to complete clot formation and contraction, respectively.
5. Remove cellular material by centrifugation at 12000 x g for 10 minutes at 4^{o}C, and store the serum in 1 ml aliquots at -20^{o}C.

Note 2: Complete emulsification is very important for a successful protocol. Routinely, this is checked by leaving the sample to stand for at least 30 minutes at room temperature and ensuring that the adjuvant and conjugate do not separate into two layers. If this occurs further mixing is required and should be retested prior to inoculation.

Note 3: In order to ensure that both inoculation and bleeding procedures are stress-free, rabbits are withstrained gently by an experienced handler in a dedicated clean lab coat with holes cut out for ears to protrude, and back legs supported to ensure the animals do not injure themselves. Rabbits are routinely housed in small groups in large cages or free-range, the latter being more stress-free.

In order to improve the quality and specificity of the antibodies, purification is recommended, particularly for use in immunohistochemical and quantitative immunoblotting experiments.

Affinity Purification

Coupling of Peptides to Sepharose Beads

The method is carried out largely as described by Chazot et al., 1999 and is used to couple the peptide to activated thiol-sepharose beads via its C-terminal cysteine residue.

1. Allow 0.35g of activated thiol-sepharose to swell in 100 ml distilled water for 15 minutes at room temperature. Place the swollen sepharose beads in a 25 ml column containing a scinter filter and wash with 100 ml of 0.1 M Tris-HCL pH 8.0, containing 0.3 M NaCl and 1mM EDTA.
2. Drain the column until 0.7 ml of buffer remained. Dissolve 1 ml of 5 mg/ml peptide in the 0.1 M Tris-HCL pH 8.0, containing 0.3 M NaCl and 1mM EDTAand add to the swollen sepharose beads and incubate for 2 hours at room temperature with gentle agitation.
3. Terminate the reaction by draining the column and washing the sepharose beads with 25 ml of Tris pH 8.0 followed by 10 ml of 0.1 M citric acid pH 4.5. All remaining un-reacted thiol groups on the sepharose beads are blocked by incubation with 3 ml of 1 mM β-mercaptoethanol in 0.1 M citric acid pH 4.5 for 1 hour at room temperature with gentle agitation.
4. Terminate the blocking reaction by washing the sepharose beads with 25 ml of 0.1 M citric acid pH 4.5. Finally equilibrate the sepharose column with 25 ml PBS and store in 10 ml PBS containing 0.02% (w/v) sodium azide, at 4^{o}C until required.

Peptide Affinity Purification of Antibodies

Purification of the anti-peptide polyclonal antibody requires the use of a 1 ml sepharose column linked to the appropriate peptide (5 mg) as described above.

1. Equilibrate the column with 100 ml of PBS, and drain. Then apply 4 ml of the immune serum to the column followed by either a 2 hour incubation period at room temperature or overnight at 4^{o}C with gentle agitation.
2. Drain unbound immune serum from the column (**Note 4**), and then wash the column slowly with 100 ml of PBS. Elute the bound antibody from the column with 8 ml of 50 mM glycine/HCL pH 2.3. Collect the eluate in 8 X 1 ml fractions, containing 15 µl of 1 M Tris to neutralise the contents to a final pH of approx. 7.4. For each fraction determine the O.D. at λ = 280 nm using standard

spectrophotometer, and pool the peak fractions (at least 0.1 OD unit); The protein concentration can be calculated using the Beer Lambert law,

C = A / ЄL

Where,

C, is the protein concentration of the antibody

A, is the absorbance at λ = 280 nm

L, is the sample path length = 1 cm.

Є, is the molar absorptivity or extinction coefficient of the chromophore at wavelength λ (the optical density of a 1 cm thick sample of a 1 M solution). Є is a property of the material and the solvent = 1.35

3. Pool the fractions containing the highest protein concentrations and dialyse with strirring against 500 ml TBS or PBS, overnight at 4^{o}C.
4. Regenerate the affinity column with 100 ml of TBS and store in 10 ml TBS containing 0.02% (w/v) sodium azide at 4^{o}C. Affinity columns can be kept for many years without losing their ability to purify antibodies.

Note 4: Retain the unbound fraction and store in case the affinity column is overloaded and does not retain all the available antibody. This is rare but does happen occasionally.

Validation Methods

Transfection methods for individual H_3R isoform expression studies

Cell culture and transfection of Human Embryonic Kidney (HEK) 293 cells

Preparation of DMEM/F12 media

All procedures should be performed under sterile conditions.

1. Mix powdered Dulbecco's Modified Eagle Medium/F12 (DMEM/F12 1:1 ratio) (15g/L) and 15mM N-2-hydroxyethylpoiperazine-N'-2-ethane sulphonic acid (HEPES) with 800 ml of sterile water. Supplement the media with 40 ml of 10% (v/v) foetal calf serum (FCS), 7.5 % (w/v) $NaHCO_3$ giving a final concentration 3.0g/L, and 20 ml of penicillin (500 µg/ml)/ streptomycin (500 µg/ml) solution.
2. Adjust the pH of the media to pH 7.6 using 10M NaOH and the final volume made up to 1 L using sterile ddH_2O. Filter-sterilise the media using a 0.2 µm Sartorius Satolab-V150 filter unit, and then store at 4^{o}C.

Sub-culturing of HEK 293 Cells

Perform all procedures under sterile conditions.

HEK 293 cells are grown in 250 ml Greiner flasks containing DMEM/F12 (as described above). The flasks are incubated in a Sanyo incubator at 37ºC, humidified in 5% CO_2. Every seven days the cells are sub-cultured.

1. 30 minutes before, prewarm sterile PBS, DMEM/F12 media and trypsin-EDTA to 37ºC.
2. Remove the old medium from the cells and wash the cells in 10 ml of pre-warmed PBS.
3. Remove the PBS and incubate the cells for 2 min in 2ml of trypsin-EDTA, at 37ºC.
4. Re-suspend the cells in 10 ml of fresh pre-warmed DMEM/F12 media by gentle pipetting up and down.
5. Add 2 ml of the cell suspension to a fresh sterile flask with a further 10 ml of fresh media and return back to the CO_2 incubator.

Polyethyleneimine (PEI) transfection method

Mammalian cells can be transfected efficiently (up to 30%) using several different techniques, including calcium precipitation, and methods using polyethyleneimine or lipofectamine (the former two being relatively inexpensive if finances are tight). Herein the polyethyleneimine (PEI) method for the transfection of HEK 293 cells adapted from **(11)**, for a 3 ml petri dish of cells (approx. 80% confluent) is described.

1. Dilute 1 µg cDNA (pCIS Flag-tagged hH_3R isoform, **Note 5**) in 100 µl 0.9% NaCl in a 1.5ml eppendorf, then add 2 µl of 1 mg/ml PEI (linear MW ~ 25,000) and flick gently to mix the contents.
2. Incubate the cDNA/PEI mixture freestanding for 10 minutes at room temperature. During the incubation period, remove the old media from the HEK 293 cells and add 2 ml of fresh pre-warmed media.
3. Following the incubation period, add the 1 ug DNA/PEI mixture to the HEK 293 cells gently mix and incubate at 37ºC in a standard CO_2 incubator.

Note 5: This CMV promoter cDNA construct Ideal for expression in mammalian cells) was a kind gift from (5), epitope tagged with a FLAG sequence which offers an alternative method of detection using commercial anti-FLAG antibodies (Sigma, UK)

Harvesting cells and membrane preparation of HEK 293 cells

1. 48 hours post transfection, remove/aspirate the culture media and replace with 1 ml of homogenisation buffer.
2. Scrape cells from the bottom of the petri dish using a Greiner cell scraper. Homogenise the re-suspended cells using a dounce glass/glass homogeniser, kept ice cold.
3. Pellet the homogenate by centrifugation at 18000 x g at 4^{o}C for 5 minutes.
4. Discard the supernatant and re-suspend the pellet in 1 ml of ice cold homogenisation buffer.
5. Re-homogenise the membranes and divide in 100 µl aliquots and store at -20^{o}C.

Immunoblots

One of the standard methods to utilise the antibodies is immunoblotting. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is commonly carried out using either 7.5 (v/v) or 10% (v/v) polyacrylamide slab gels (appropriate for analysing monomeric (30-45kDa) and dimeric species (75-90kDa)) under reducing conditions. Lower percentage polyacrylamide can be used for higher molecular weight proteins. Immunoblots can then be probed either with either anti-FLAG antibody (commercial, Sigma) in the case of the FLAG tagged human H_3R clones, or with the appropriate rabbit anti-H_3R antibodies (untagged human H_3R clones).

Preparation of resolving gel

1. Recipe for Resolving gel is as follows:
2. Prepare the resolving gel by mixing 6 ml of double-distilled water with 3 ml of resolving gel buffer (50 mM Tris, 384 mM glycine, 1.8 mM EDTA and 0.1% (w/v) SDS pH 8.8), 6 µl of TEMED, 3 ml of stock 30% acrylamide, and 60 µl 10% (w/v) ammonium persulphate (APS). Immediately pour the polyacrylamide solution gently into a pre-assembled Hoefer SE 245 dual gel caster, using two gel plates of 10 x 8cm and spacers of 1 mm width.
3. Layer 100 µl of saturated water/ butanol solution over the top of each gel. Cover the gels with parafilm and allow to polymerise for 40 - 60 minutes at room temperature. The gels can be used immediately or stored by wrapping the gels individually in tissue paper and stored in electrode buffer (50 mM Tris, 384 mM glycine, 1.8 mM EDTA and 0.1% (w/v) SDS pH 8.8.) at 4^{o}C until required.

Preparation of protein samples for SDS-PAGE

Protein samples for SDS-PAGE are precipitated using the chloroform/ methanol precipitation.

1. To the protein sample (25-50 µg), add 4 x volumes of ice-cold methanol and vortex-mix samples and centrifuge at room temperature at 18,000 x g for 1 minute.
2. Add 1 x volume of ice-cold chloroform to the samples, vortex-mix and centrifuge at room temperature at 18,000 x g for 1 minute.
3. Add 3 x volumes of ice-cold water to the samples, vortex mix and centrifuge at room temperature at 18,000 x g for 1 minute.
4. Carefully discard the upper layer and add 1x volume ice-cold methanol to the samples, vortex-mix and centrifuge at room temperature at 18,000 x g for 4 minute.
5. Discard the supernatant and ari-dry the pellet. Re-suspend the dried protein pellet by vortex mixing in 5 µl of sample buffer (30 mM sodium hydrogen phosphate, pH 7.0, 30% (v/v) glycerol, 0.05% (v/v) bromophenol blue and 7.5% (w/v) SDS), 2 µl of 200mM DTT and 8 µl of water to a final volume of 15 µl.
6. Boil the samples at 95oC in the heat block for 5 minutes and then centrifuge at 18,000 x g for 30 seconds at room temperature before analysis by SDS-PAGE.

SDS-PAGE

1. Carefully clamp the resolving mini-slab acrylamide gel into a Hoefer mini-vertical gel electrophoresis unit SE260.
2. Prepare the stacking gel by mixing 2.3 ml of water with 1 ml of stacking gel buffer (0.5 M Tris-glycine, pH 6.8, containing 8 mM EDTA and 0.4% (w/v) SDS), 650 µl of stock 30% acrylamide, 5 µl of TEMED and 80 µl of 10% (w/v) ammonium persulphate.
3. Pour the stacking gel mixture immediately and gently (avoiding air bubbles) into the mini-slab gel on top of the resolving gel and insert a welled comb into the stacking gel. Polymersation takes up to 10 minutes.
4. Check and carefully remove the comb and wash the the wells with double-distilled water.
5. Pour approximately 300 ml of electrode buffer (see above) into and behind the wells and into the base of the electrophoresis unit.

6. Load using a Hamilton syringe or pipette with gel loading tip extansion 15 µl of Protein samples and pre-stained standards (protein molecular weight range of 200 – 6.5 kDa) into the ten wells of the stacking gel.Ensure all lanes are loaded.
7. Perform electrophoresis at a constant current (10 mA (per gel) initially and then increase the current by 5 mA or 10mA (per gel) once the samples reached the resolving gel. Gel run time is approximately 2 hours or until the appropriate pre-stained molecular weight marker (25 kDa) are at the bottom of the resolving gel.

Immunoblotting stage

After SDS-PAGE is complete, the proteins from the gels are transferred to a nitrocellulose membrane.

1. Recipe for Transfer buffer is as follows: 25 mM Tris, pH 8.4, 192 mM glycine and 20% (v/v) methanol.
2. In order to do this, construct a transfer cassette in the following order of components each of which had been pre-equilibrated in transfer buffer sponge, two sheets of blotting paper, nitrocellulose membrane, SDS-PAGE gel, two sheets of blotting paper and finally a piece of sponge.
3. On the addition of each component to the transfer cassette, remove air bubbles carefully using a glass pipette or plastic tube. Transfer proteins at a constant voltage of 50 V for 2 hours using a Hoefer TE 22 tank transfer unit containing transfer buffer kept cool with external ice packs.
4. Following the transfer of the proteins, briefly rinse the nitrocellulose and incubate with 15 ml of blocking buffer, which comprises of PBS, containing 5% (w/v) dried milk and 0.02% (v/v) Tween-20, for 1 hour at room temperature with gentle agitation.
5. After blocking of the non-specific antibody sites, wash the nitrocellulose membrane with 10 ml of PBS. Dilute the appropriate affinity-purified primary antibodies in incubation buffer, which comprises of PBS, containing 2.5% (w/v) dried milk to working concentrations of (0.25 – 5 µg purified antibody/ml).
6. Incubate the nitrocellulose membranes with 10 ml of the diluted primary antibody solution for 2 hours at room temperature, or overnight at 4^{o}C with gentle agitation (petri dish or plate).
7. After incubation with the primary antibody, wash the nitrocellulose membranes four occasions in 10 ml of wash buffer, consisting of PBS, containing 2.5% (w/v) dried milk and 0.2% (v/v) Tween-20, for 10 minute intervals with gentle agitation on a shaker at room temperature.

8. Incubate the nitrocellulose membranes for 1 hour with gentle agitation with horseradish peroxidise (HRP) labelled secondary antibody, either anti-rabbit or anti-mouse depending on the host species for primary antibody, at a dilution of 1/2000 in 10 ml of incubation buffer.
9. Remove the unbound secondary antibody by washing the membrane as detailed above.
10. Drain the nitrocellulose and briefly rinse with PBS
11. Develop the immunoreactive bands on the nitrocellulose membrane by processing in a solution containing, 100 µl of 68mM p-coumaric acid, 10 ml of 1.25 mM luminal and 6 µl of 30% (v/v) H_2O_2, for 1 minute at room temperature (**note 6**).
12. After incubation, wrap the immunoblot in cling film, and place in a film cassette.
13. Expose the immunoblots to Hyperfilm^{TM} for various times between 1-5 minutes, and develop the film developed in Kodak D-19 Developer until the immunoreactive bands are visible and fix in Kodak Unifix for 5 minutes at room temperature.

Note 6: This is a homemade recipe developed in the laboratory. It has a drawback in that it lacks a stabiliser and has a use limit of aprox. 10 minutes. There are a number of commercial products which can be used which contain stabilisers and can be utilised for longer.

Immunohistochemical analysis

Immunohistochemical analysis is carried out as essentially described previously by Chazot et al. (2001); Cannon et al. (2007).

Diaminobenzidine IHC

1. Perfusion-fix adult mouse brains with 4% (w/v) paraformaldehyde (0.05% (v/v) glutaraldehyde in 0.1M phosphate buffer, pH 7.4.
2. Remove brains, post-fixin 4% (w/v) paraformaldehyde (0.05% (v/v) glutaraldehyde in 0.1M phosphate buffer, pH 7.4overnight and then cryoprotect the tissue by incubation in 10%, 20% and 30% (w/v) sucrose in 0.01 M phosphate buffer, pH 7.4 at 4^{o}C for 48 hours in an even step-wise-manner.
3. Freeze the tissue at -80^{o}C in isopentane for 1.5 minutes, and cut coronal sections (25 µm thick) on a cryostat set to -20 ^{o}C. Pick up sections using a fine paintbrush and store in PBS (with 0.05% sodium azide) for no more than 2 weeks at 4 ^{o}C.

4. Treat free-floating sections initially with 10% (v/v) methanol and 3% (v/v) hydrogen peroxide in 50 mM TBS, pH7.4, for 10 minutes to quench endogenous peroxidise activity.
5. Incubate sections in PBS, 0.2% (w/v) glycine and 0.2% (v/v) Tween-20, for 15 minutes to mop up residual un-reacted aldehyde groups from the fixative.
6. Block non-specific antibody binding sites on the tissue by incubating with 2% (v/v) foetal calf serum in PBS, 0.02% (v/v) Tween-20 for 1 hour. S
7. Incubate sections overnight at 4^{o}C in the primary anti-H_3R antibody at a range of concentrations (0 – 5 µg/ml) in 1% (v/v) foetal calf serum/ PBS.
8. After washing the sections on threeoccasions in PBS/ 0.1% (v/v) Triton X 100, detect antibody binding using the Vectastain ABC Elite kit.
9. Incubate the sections with a biotin linked, anti-rabbit secondary antibody for 2 hours followed by incubation for 1 hour with streptavidin-horse radish peroxidase (HRP) complex.
10. Visualise the immune reaction using 3,3'-diaminobenzidine tetrahydrochloride as the HRP substrate.

Immunofluorescence protocol

1. Perfuse-fix adult mouse brains with 4% (w/v) paraformaldehydein 0.1M phosphate buffer, pH 7.4.
2. Remove brains, post-fixin 4% (w/v) paraformaldehyde in 0.1M phosphate buffer, pH 7.4overnight and then cryoprotect the tissue by incubation in 10%, 20% and 30% (w/v) sucrose in 0.01 M phosphate buffer, pH 7.4 at 4^{o}C for 48 hours in an even step-wise-manner.
3. Freeze the tissue at -80^{o}C in isopentane for 1.5 minutes, and cut coronal (or other appropriate orientation) sections (14 µm thick) on a cryostat set to -20 ^{o}C.Free floating sections may be collected as above, alternatively if thinner sections are preferred (8-16 µm), they can be collected onto poly-L-lysine coated slides, air dried and stored at -80^{o}C in air tight boxes until use.
4. On the day of the assay remove the slides from the freezer and air dry again for one hour.
5. Block the sections for 45 mins at room temperature in PBS pH 7.4 containing 0.4% Triton X-100, 4% foetal calf serum (FCS) or normal serum from the same species as the secondary antibodies are raised in and 1% bovine serum albumin (BSA).
6. Following the blocking stage rinse the sections briefly in PBS and thenincubate for 48 hrs at 4^{o}C with primary anti-H_3R antibody at a range of concentrations (0

– 5 µg/ml).The antibody diluent for both the primary and secondary antibodies is PBS pH 7.4 containing 0.1% Triton X-100, 1% FCS (or normal serum as before) and 1% BSA.

7. Rinse sections and wash in PBS (3 x 10 mins), and then apply secondary antibodies:fluorophore linked anti-rabbit antibodies e.g. Cy3 or Alexa 488, for 1 hour.
8. Rinse sections and wash in PBS as before and mount in Citifluor AF4 or equivalent.

Controls should include the omission of the primary antibody and a peptide block where the primary antibody is pre-incubated with the relevant peptide immunogen (100-fold excess) to confirm antibody specificity.

Peptide block to confirm antibody specificity for individual immunogen

In order to confirm that immunoreactivity detected either in the immunoblots or in immunohistochemical analysis is specific to the amino acid sequence of the immunizing peptide, a peptide block can be carried out.

1. Pre-incubate the antibody overnight at 4^{o}C with an equal volume of the relevant peptide. The peptide is used in excess (500 µg/ml) to ensure complete blockade of antibody binding.
2. Adjust the final antibody dilution to take into account the initial 1:2 dilution with the peptide.

Any immunoreactive bands or staining which persists after the antibody block is considered to be due to non-specific antibody binding. Anti-human H_3R isoform specific antibodies have been validated against HEK 293 cells transfected with the respective H_3R isoform cDNA. The next set of results looks at the validation of each of the antibodies.

Representative examples of results for specificity demonstration and control experiments

Isoform specificity of the anti-r$H_{3A/C}$/ h$H_3R_{445/453}$(268-277) antibody

The sequence chosen is specific to third intracellular loop region of the rat H_{3C} isoform. The antibody is shown to be selective for the rat H_{3A}, rat H_{3C}, but not rat H_{3B} (not shown). Herein, it is shown that the antibody in terms of human isoform also detects both the full length human $H_3R_{445}$and H_3R_{453}isoforms (Figure 4). The H_3R_{453} isoform is the result of an additional exon utilised resulting in an additional 8 amino acids added

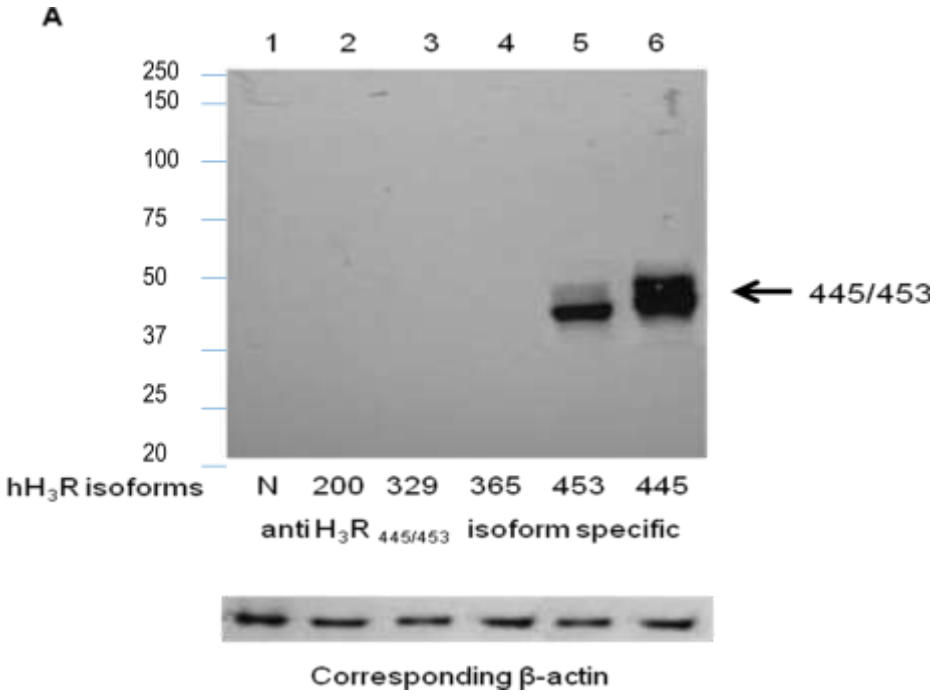
at the C-terminus (Wellendorph et al., 2002). The significance of this very rare isoform is yet to be determined.

Immunoblot showing labelling of the human $H_3R_{445/453}$ isoforms using the anti-rH_3R_{AC} / $hH_3R_{445/453}$ antibody:

Ab 1. Isoform specificity of the rH_{3C} (268 – 277) antibody

rH_{3C} (268 – 277) EAMPLHRGSK-Cys

Sequence specific to the i3 region in the rat H_{3C} isoform. This antibody was shown to be selective for the rat H_{3A} and rat H_{3C} isoforms and, surprisingly only the full length human $H_{3(445/453)}$ isoform (Fig 4). Antibodies purified from this sequence in different rabbits and different bleeds consistently showed the same human $H_{3\ (445)}$ selectivity (**13)**



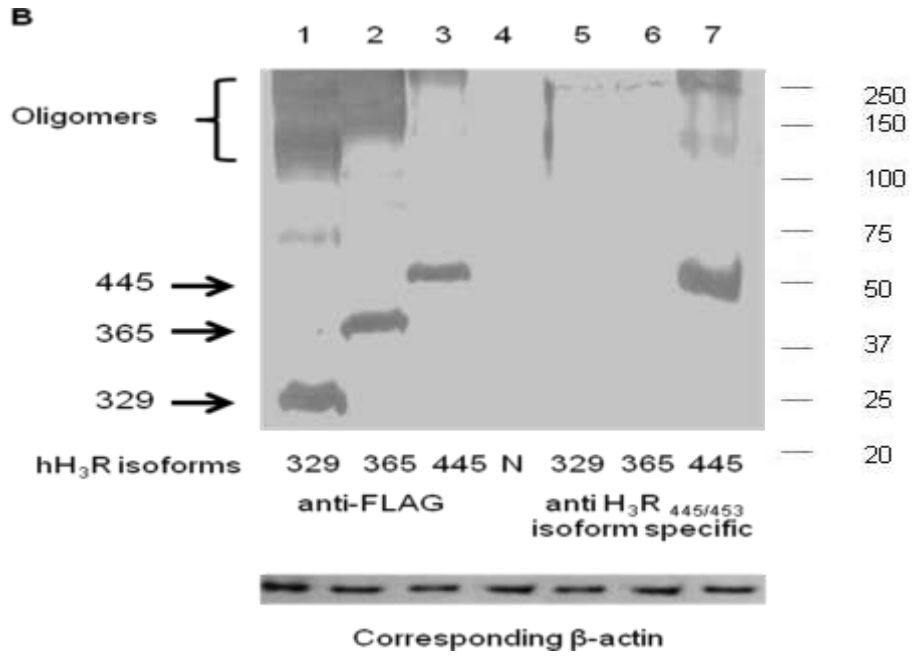


Figure 4 (A) Immunoblot of five different human H_3R isoforms probed with anti-rH_{3AC}/ $hH_3R_{445/453}$ specific antibody.

Approximately 25 µg of protein/ well were loaded onto a 7.5% PAGE gel. Once transferred the membrane was probed with affinity purified anti-rH_{3AC}/ $hH_3R_{445/453}$ antibody (1 µg/ml concentration). The antibody detects only the full length hH_3R 445 and 453 isoforms with no cross reactivity with the shorter isoforms. The lower panel shows the corresponding β-actin, probed with monoclonal mouse anti β-actin antibody (1:5000). Lane 1, HEK 293 cells untransfected but still contain PEI; Lane 2, HEK 293 cells expressing hH_3R_{200}; Lane 3, HEK 293 cells expressing hH_3R_{329}; Lane 4, HEK 293 cells expressing hH_3R_{365}; Lane 5, HEK 293 cells expressing hH_3R_{453}; Lane 6, HEK 293 cells expressing hH_3R_{445}.

Figure 4 (B) Immunoblot of three different FLAG tagged human H_3R isoforms probed with anti-FLAG (lanes 1-3) and the anti-rH_{3AC}/ $hH_3R_{445/453}$ specific antibody (lanes 4-7).

Homogenates of HEK 293 cells transfected with three different human H_3R isoforms (329, 365 and 445), all epitope tagged with FLAG were prepared. Approximately 25 µg of protein/ well were loaded onto a 7.5% PAGE gel. Identical sample were run on both left and right hand sides of the gel. Once transferred onto a nitrocellulose membrane, the left side panel was probed with a monoclonal mouse anti-FLAG antibody (1:5000 dilution) while the right side panel probed with affinity purified anti-rH_{3AC}/ $hH_3R_{445/453}$ antibody (1 µg/ml concentration). The FLAG antibody reacts with all three isoforms with monomeric species migrating at approximately Mr 33, 36, 44 kDa. The anti-rH_{3AC}/ $hH_3R_{445/453}$ antibody detects only the full length 445 isoform with no cross reactivity with

the two shorter isoforms. Lower panel shows corresponding β-actin signal, probed with monoclonal mouse anti β-actin antibody (1:5000). Lanes 1 and 5, HEK 293 cells expressing hH_3R_{329}; Lanes 2 and 6, HEK 293 cells expressing hH_3R_{365}; Lanes 3 and 7, HEK 293 cells expressing hH_3R_{445}; Lanes 4, HEK 293 cells untransfected but still contain PEI. All blots shown are representative blots from at least 8 similar experiments.

Once the reactivity of antibody against the human H_3R isoform transfected cells was determined, the selectivity of the antibody was determined (Fig 5).

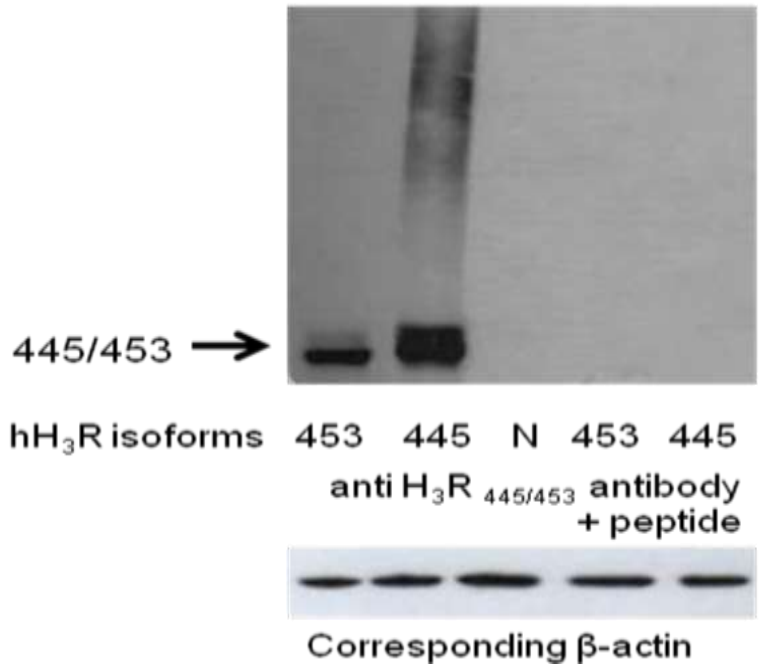


Figure 5: Selectivity of the anti-rH_{3AC}/ $hH_3R_{445/453}$ antibody using the corresponding peptide sequence:

Immunoblot confirming the selectivity of the anti-rH_{3AC} / $hH_3R_{445/453}$ antibody. Approximately 25 µg of protein/ well were loaded onto a 7.5% PAGE gel. Identical samples were run on both left and right hand sides of the gel. Once transferred onto a nitrocellulose membrane, the left side was probed with the affinity purified anti-rH_{3AC}/ $hH_3R_{445/453}$ antibody (1µg/ml concentration) while the right side was probed with affinity purified anti-rH_{3AC}/ $hH_3R_{445/453}$ antibody pre-incubated with the antigen peptide (1 µg/ml concentration).

The major immunoreactive bands labelled in HEK 293 expressing the hH_3R_{453} or hH_3R_{445} (lanes 1 and 2, respectively) were greatly suppressed by pre-incubation with the

antigen peptide (lanes 4 and 5), demonstrating the sequence selectivity of the antibody. Lane 3, HEK 293 cells mock transfected. Lower panel shows corresponding β-actin, probed with monoclonal mouse anti β-actin antibody (1:5000). The anti-rH_3R_{AC}/ h$H_3R_{445/453}$ antibody has been shown to detect only the full length human and rat isoforms as well as a truncated rH_3R_C isoform. This antibody is useful for looking at the full length H_3R in both rodent and human tissue.

Occasionally, this rationale directed approach yields unexpected results. One such example is the following antibody where an attempt was made to generate a anti-hH_3R_{365} selective antibody. However, when screened it was found to only selectively identify the hH_3R445 isoform.

Isoform specificity of the anti-hH_3R_{365} peptide 2 (268-278) antibody

The sequence chosen is specific to the third intracellular region in the human H_3R_{365} isoform. This antibody was shown to be selective for the human H_3R_{445} isoform only (Figure 6).

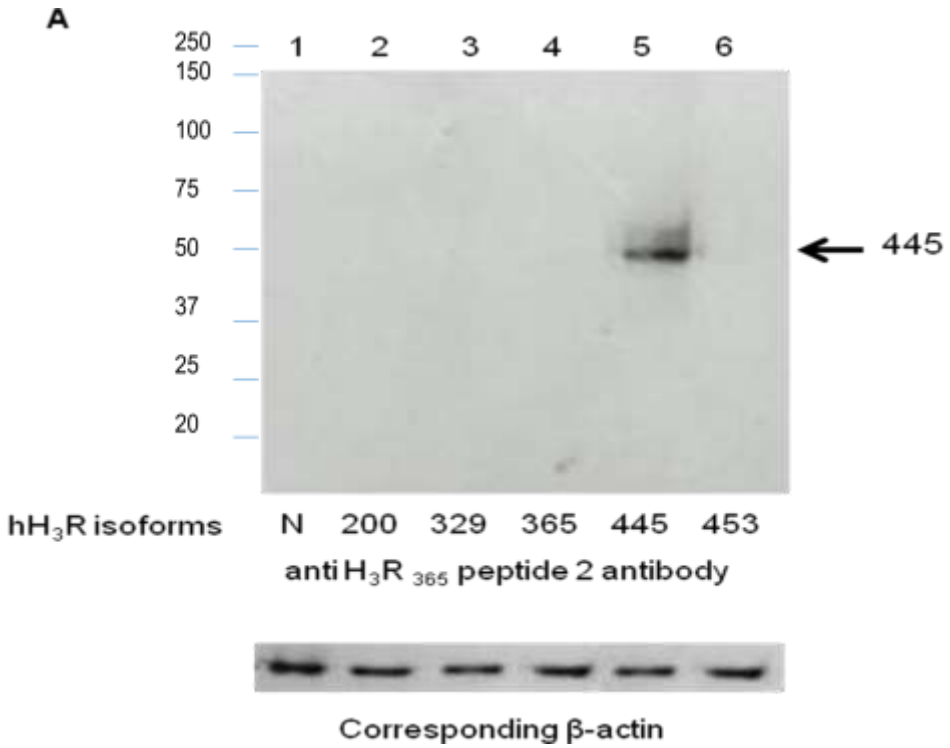


Figure 6 Immunoblot of five different human H_3R isoforms probed with anti-hH_3R_{365} peptide 2 specific antibody.

Approximately 25 µg of protein/ well were loaded onto a 7.5% PAGE gel. Once transferred the membrane was probed with affinity purified anti-hH_3R_{329} antibody (3 µg/ml concentration). The anti-hH_3R_{329} antibody detects only the H_3R 329 isoform. No cross reactivity with the other shorter or longer isoforms was detected. Lower panel shows the corresponding β-actin, probed with monoclonal mouse anti β-actin antibody (1:5000). Lane 1, HEK 293 cells mock transfected; Lane 2, HEK 293 cells expressing hH_3R_{200}; Lane 3, HEK 293 cells expressing hH_3R_{329}; Lane 4, HEK 293 cells expressing hH_3R_{365}; Lane 5, HEK 293 cells expressing hH_3R_{445}; Lane 6,HEK 293 cells expressing hH_3R_{453}.

In contrast, the following antibody was found to selectively label the isoform to which it was directed, namely the hH_3R_{329} isoform (Figure 7).

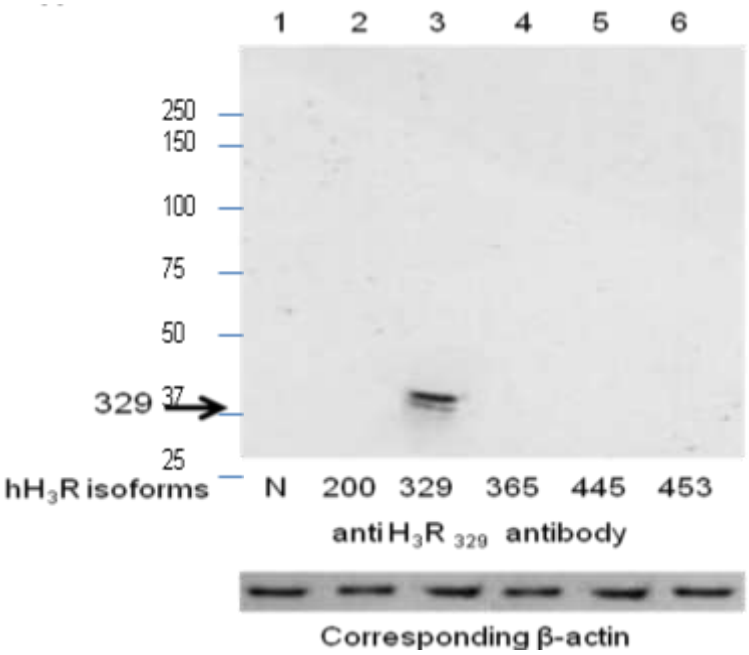


Figure 7 Immunoblot of five different human H_3R isoforms probed with anti-hH_3R_{329} specific antibody.

Approximately 25 µg of protein/ well were loaded onto a 7.5% PAGE gel. Once transferred the membrane was probed with affinity purified anti-hH_3R_{329} antibody (3 µg/ml concentration). The anti-hH_3R_{329} antibody detects only the H_3R 329 isoform. No cross reactivity with the other shorter or longer isoforms was detected. Lower panel shows the corresponding β-actin, probed with monoclonal mouse anti β-actin antibody (1:5000). Lane 1, HEK 293 cells mock transfected; Lane 2, HEK 293 cells expressing

hH_3R_{200}; Lane 3, HEK 293 cells expressing hH_3R_{329}; Lane 4, HEK 293 cells expressing hH_3R_{365}; Lane 5, HEK 293 cells expressing hH_3R_{445}; Lane 6,HEK 293 cells expressing hH_3R_{453}.

Isoform specificity of the H_3R_{200}/ isoform 5 (191-200) antibody

The sequence was generated to the C terminus of the human H_3R_{200} because of its unique C terminal sequence. The antibody was shown to detect immunoreactivity in both human putamen (not shown) and recombinant HEK 293 cells expressing the H_3R_{200} cDNA (Fig 8).

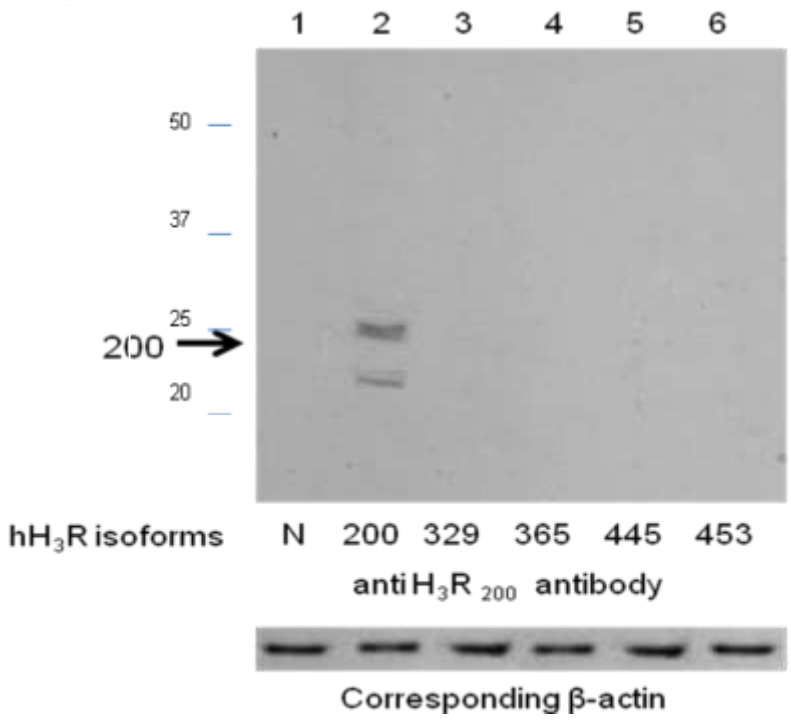


Figure 8 Immunoblot of five different human H_3R isoforms probed with anti-hH_3R_{200} specific antibody.

Approximately 25 µg of protein/ well were loaded onto a 10% PAGE gel. Once transferred the membrane was probed with affinity purified anti-hH_3R_{200} antibody (3 µg/ml concentration). The anti-hH_3R_{200} antibody detects only the 200 isoform. No cross reactivity with the longer isoforms was detected. Lower panel shows the corresponding β-actin, probed with monoclonal mouse anti β-actin antibody (1:5000). Lane 1, HEK 293 cells mock transfected; Lane 2, HEK 293 cells expressing hH_3R_{200}; Lane 3, HEK 293 cells expressing hH_3R_{329}; Lane 4, HEK 293 cells expressing hH_3R_{365}; Lane 5, HEK 293 cells expressing hH_3R_{445}; Lane 6, HEK 293 cells expressing hH_3R_{453}.

***V*alidation studies using H_3R KO mice**

Immunoblotting (IB) studies in mice

As a representative, the specificity of the **anti-$rH_{3A/C}$/ anti-$hH_{3(445)}$** antibody for murine H_3Rs was confirmed by using it to probe immunoblots of (H_3R +/+) wild type (WT) and (H_3R -/-) knockout (KO) mouse brain. Two bands at Mr 50,000 (± 2000) and Mr 44,000 (± 2000) were consistently detected in WT mouse brain but not in the KO material. Occasionally a Mr 40,000 species was also selectively detected (Figure 9).

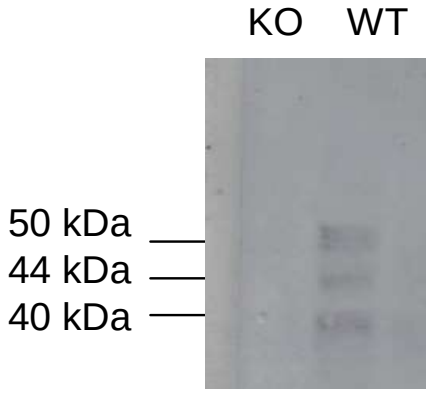


Fig 9 Immunoblot using anti-$rH_{3A/C}$/ anti-$hH_{3\ (445)}$ to compare H_3 receptor immunoreactivity in H_3R -/- knockout (KO) mice with H_3R +/+ wild type (WT) mice
KO and WT mouse forebrain (10 µg protein) was probed in parallel with anti-$rH_{3A/C}$/ $hH_{3\ (445)}$ R used at 1µg/ml. Two bands at Mr 50,000 ± 2000 and 44,000 ± 2000 were consistently detected in WT but not in KO material (n = 3 separate mice). After each immunoblot the nitrocellulose sheet was stained with Ponceau S to confirm that the total protein loading was the same for both the KO and WT lanes.

Immunohistochemical (IHC) studies in the mouse

Immunohistochemical studies using anti-$rH_{3A/C}$/ anti-$hH_{3(445)}$ antibody to compare immunoreactivity in H_3R -/- (KO) mice with wild type (WT) mice clearly demonstrated the specificity of this antibody. The anti-$rH_{3A/C}$/$hH_{3(445)}$ isoform specific antibody was used to carry out a detailed mapping of the anatomical distribution of the H_3R in mouse brain. IHC was carried out in wild type (H_3R +/+) mouse brain slices alongside identical slices obtained from (H_3R -/-) animals. Additional controls included the prior incubation of the anti-$hH_{3\ (445)}$ antibody with its respective peptide such that specific immunoreactivity would be blocked. To achieve this, the primary antibody was incubated with the peptide at a final concentration of 500µg/ml overnight at 4°C. Before use the antibody/peptide mixture was diluted such that the final antibody concentration

was the same as for the unblocked antibody. As a second control IHC was carried out with the secondary antibody alone.

A descriptive summary of the immunoreactivity in selected brain regions is shown in Table 1 for both wild type and H_3R -/- mice. Table 1 a) and b) show results using the pan anti-H_3R (346-358) antibody and the isoform specific anti-r$H_{3A/C}$***/*** anti-h$H_{3(445)}$ antibody, respectively. The data are from three different mice and the findings are in agreement with those from an independent laboratory using our antibodies to screen at least four further WT and KO animals (Dr Keri Cannon, Johnson & Johnson). Intensity of staining has been graded 0 – 4, with 0 being equivalent to background and 4 representing intense labelling. The strongest specific staining (that is not present in knockout animals) was seen in cortical laminae II, somatosensory cortex, piriform cortex, and the amygdaloid complex (Fig 10A). Moderate reactivity was also noted in the str terminalis, str: caudate putamen, fimbria of the hippocampus and the cingulum. Weak non-specific reactivity was observed in some of the brain structures of knockout animals using the anti-r$H_{3A/C}$/ **a**nti-h$H_{3(445)}$ antibody (Figure 10). This non-specific staining was not seen with an immunofluorescence detection system using rhodamine linked anti-rabbit secondary antibodies (Fig 10B).

The background strain of the H_3R -/- mice is C5731/6J (US Patent no. 7151200). In contrast to results using the pan anti-H_3 (346 – 358) in B6C3Fe mice **(7)** there was negligible immunostaining in the deeper cortical layers and little detectable signal above background in the CA1, CA2 and CA3 regions of the hippocampus. In B6C3Fe mouse brain slices pan anti-H_3 (346 – 358) immunoreactivity was detected in cortical layers II and V, and in the CA3 region of the hippocampus **(7).**

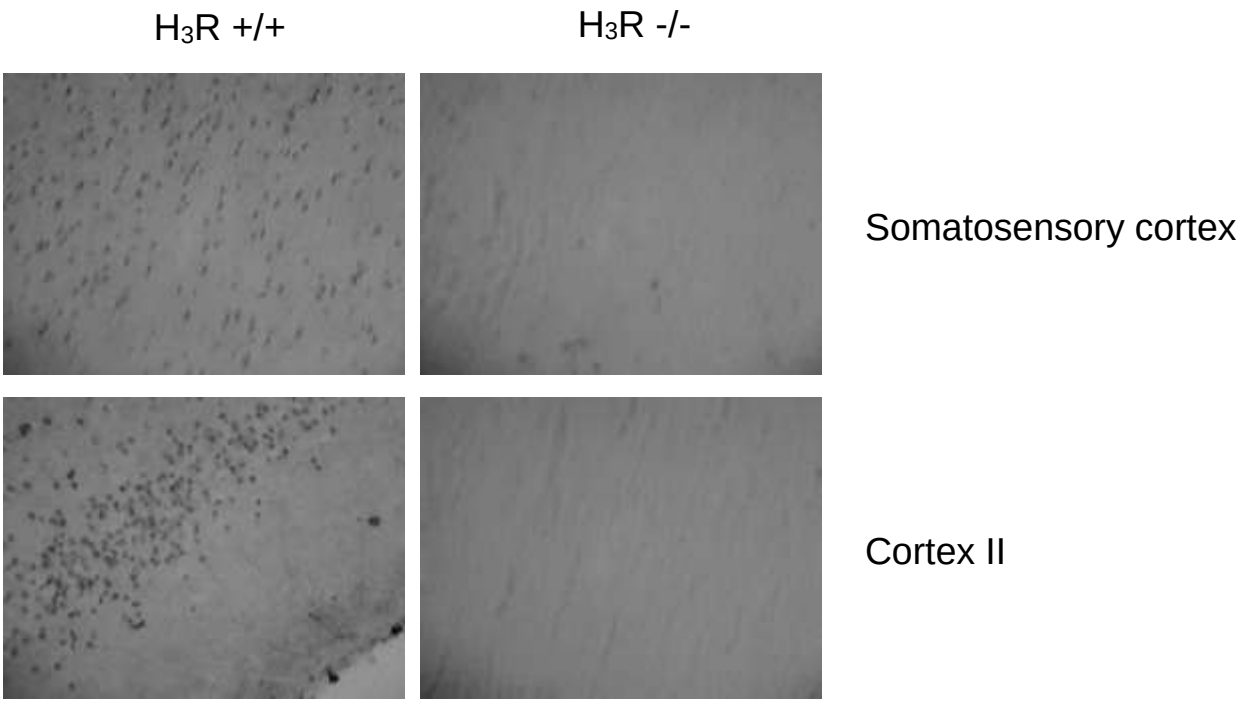
Table 1 a) Summary of some key mouse brain structures labelled using pan-H_3 receptor antibody

Tissue	**H_3R +/+**	**H_3R -/-**
Striatum	+3-4	0
Sunstantia nigra	+3	0
S1/S2 cortex	+3-4	+1
Cingulate cortex	+3	0
Entorhinal cortex	+3	0
Thalamus	+3	0

Amygdaloid complex	+3-4	0
Hippocampus CA1	0-1	0-1
Medial septal cortex	0	0

Table 1 b) Summary of some key mouse brain structures labelled using anti-$rH_{3A/3C}$ / $hH_{3(445)}$ antibody

Tissue	**H_3R +/+**	**H_3R -/-**
Substantia nigra	+4	+1
Striatum	+3	+1
Cingulum	+3	+2
Somatosensory cortex	+3	0
Fimbria of the hippocampus	+3	+2
Hippo: CA1/CA2/CA3	0	0
Cortex layer II	+4	0
Piriform cortex	+4	0
Str terminalis	+4	+1
Amygdaloid complex	+4	0



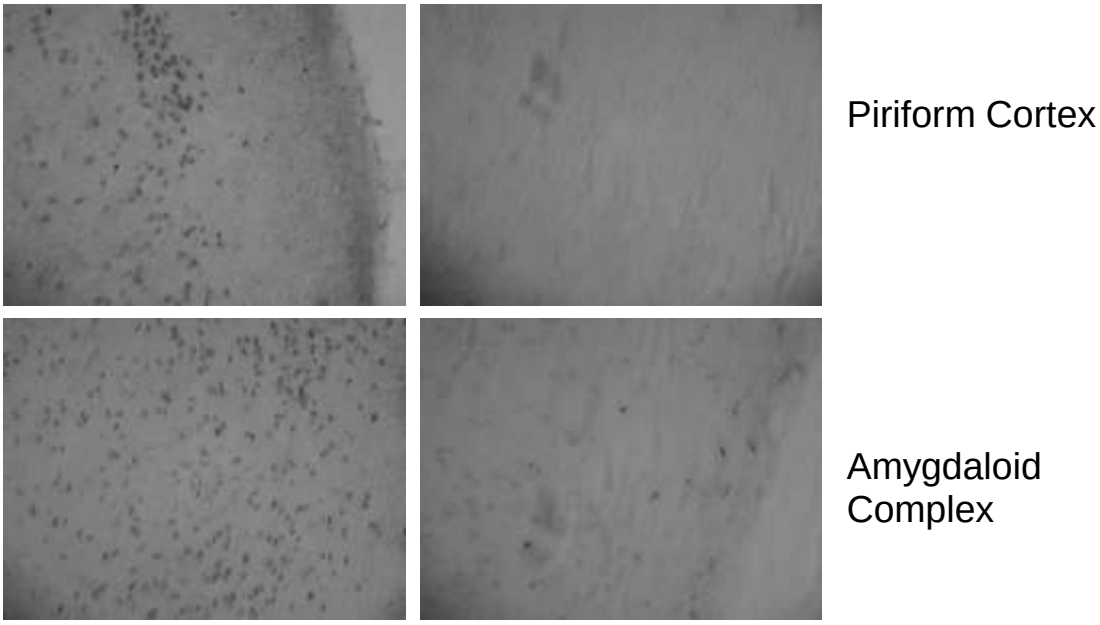


Figure 10A Immunohistochemical detection of H_3R immunoreactivity in a range of representative brain structures

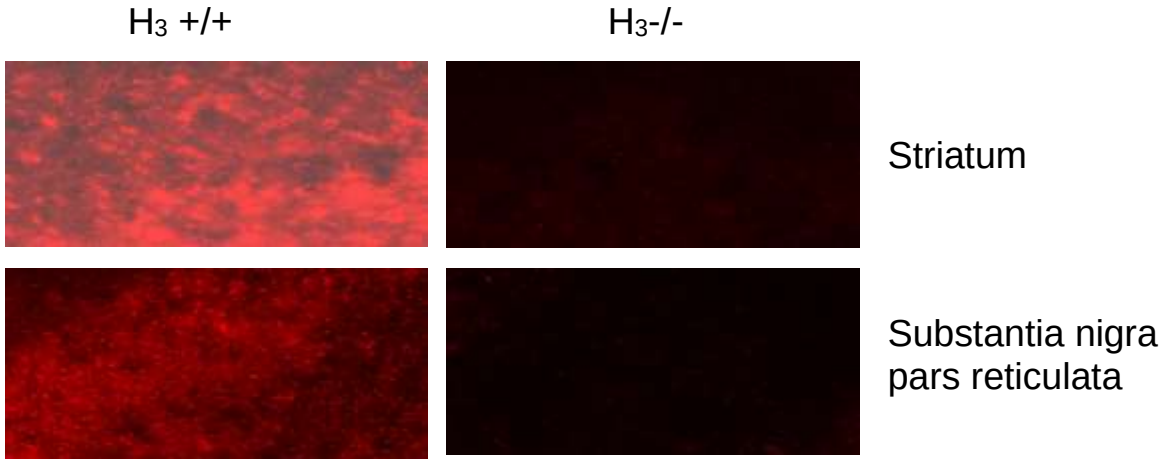


Fig 10B Immunofluorescent detection of H_3 immunoreactivity in striatum and substantia nigra pars reticulata comparing H_3 +/+ (wild type) mice with H_3 -/- (knockout) mice (*Thanks to Dr Keri Cannon) Anti-r$H_{3A/3C}$ /anti-hH_3R (445) antibody in coronal mouse brain slices.*

Conclusions

This chapter describes the development and characterization of a selection of novel anti-human H_3R isoform selective immounoprobes: These probes can be used to investigate expression of three of the human H_3R isoforms 445, 365 and 329 in native human tissue. Different isoforms have been shown for the first time to be expressed at the protein level in the human CNS (eg. **12**; **13**). Future studies can now explore whether there are changes in human H_3R isoforms expression in ageing or disease. A peptide sequence unique to the rat H_{3C} /$H_{3(397)}$ isoform was chosen and used to immunise rabbits for the generation of sequence specific antibodies. The antibody was affinity purified and screened against rat and human H_3R isoforms expressed in HEK 293 cells. Immunoblotting of cell homogenates revealed that in addition to detecting the rH_{3C} /397 isoform it also labelled the full length 445 isoforms of both the human and the rat H_3R. The peptide sequence EAMPLHRGSK is found within the third intracellular loop of the rat rH_{3C} /397 isoform, aa 268 – 277. The sequence is present in both the human and rat full length forms but in the rH_{3C} /397 isoform it is split: EAMPLHR appearing on the 5' side of the deletion site and GSK on the 3' side. Presumably the tertiary structure of the receptor is such that the two halves are brought together in a structural form which the antibody can recognize. One might have expected the antibody to react with $hH_{3\ (365)}$ (deletion 275-354) and rH_{3B} /413 (deletion 275 – 306) since only the last two amino acids are missing from the EAMPLHRGSK sequence in these two isoforms. In addition in the full length mouse H_3R the sequence is EAMPLHR**YGV**, with the last three amino acids being different from the immunising peptide, nevertheless specific anti-H_3R immunostaining was detected with both the pan anti-H_3 and the anti-$rH_{3A/3C}$ ***/***anti-$hH_3R_{\ (445)}$in mouse brain slices (validated by H_3 (-/-) mice). However, the tertiary structure of the $hH_{3\ (365)}$ and rH_{3B} /413 may have been unfavourable, with the peptide sequence presumably not accessible to the antibody within these isoforms. The $hH_{3(329\ \Delta IC)}$ (deletion 227 -342) does not possess any of this particular peptide sequence and, as expected, no reactivity was seen with this expressed clone.

Immunoblots of receptors expressed in HEK293 cells revealed the presence of higher molecular weight species in addition to bands running at the expected molecular weight of the respective receptor isoforms. The receptor is not being expressed in its natural environment and expression levels are generally considerably higher than in native tissue. However, higher molecular weight species were also observed in immunoblots of native tissue, especially in the human material. Oligomerisation of rat H_3R has been described by ourselves and others (**14,15**) and there is a growing

consensus that many GPCRs, possibly all of them, function as dimers or higher molecular weight oligomers. With rat isoform H_{3C}/397 expressed in HEK 293 cells, higher molecular weight species were less apparent than with the full length rH_{3A}/445 isoform.

Attempts were made to generate antibodies against three more human H_3R isoforms using an identical strategy. This strategy yielded in some cases unexpected results, for example Ab 2 designed to be a selective hH_3 365 ab actually yielded a H_3 445/365 specific antibody, Ab 3 designed to produce a hH_3 365 selective antibody surprisingly yielded a H_3 445 selective antibody. In contrast, Ab 4 designed to produce an anti-H_3 329 antibody, proved successful in yielding such an antibody; Finally, Ab5 directed to a unique sequence with the highly truncated H_3 200 receptor again proved successful despite the limited available selective immunogen sequence. Therefore, to date a panel of anti-H_3 200, H_3 329 and H_3 445 antibodies have been successfully generated; a mixed specificity H_3 365/H_3 445 antibody may have some utility in immunoblotting methods.

Immunohistochemical (IHC) studies using wild-type and H_3R-/- mice (Lovenberg, Johnson and Johnson, US Patent no. 7151200) showed a marked ablation in immunoreactivity in the knockout animals compared to the wild type, demonstrating the specificity of the antibodies. The immunoreactivity in wild type animals was similar for both the original pan anti-hH_3 (346-358) antibody (not shown) and the new isoform selective antibody. The findings also confirmed those of a previous study using the pan anti-H_3 (346-358) antibody **(7)** to map the anatomical distribution of the H_3R in mouse brain slices; with the exception of cortex layer V which showed moderate/strong staining in the first study but only light staining in the present investigation, and the hippocampus (regions CA1-CA3 and dentate gyrus) where there was some moderate reactivity, not found here. It is possible that these differences might be accounted for by the fact that different mouse strains were used (B6C3Fe, **(7)**; C5731/6J in the more recent study), however further work is necessary to validate these observations and confirm their reproducibility. IHC using the anti-rH_{3C}/$hH_{3\ (445)}$ antibody demonstrated clear specific immunoreactivity in substantia nigra, cortical laminae II, somatosensory cortex, piriform cortex, and the amygdaloid complex. Moderate reactivity was also noted in the striatum, *str* terminalis, fimbria of the hippocampus and the cingulum. Background labelling was evident in some brain areas in knockout animals, notably the fimbria of the hippocampus and cingulum, and to a lesser degree in the striatum; nevertheless it was at a significantly lower level than in equivalent areas from wild type

mice. This background labelling was not apparent using an immunofluorescent detection system e.g. basal ganglia (Fig 10B). A similar picture was observed in the pain circuits study with the pan-H_3R antibody; clear labelling was observed in wt mice for a subpopulation of Aδ fibres, and was completely absent in H_3R (-/-) mice; however a small subset of Aβ fibres were labelled non-specifically as evidenced by maintenance of this immunoreactivity in H_3R (-/-) mice **(8**). Furthermore, in immunoblots comparing mouse brain homogenates from WT and KO the animals there was an indication of some immunoreactivity in the KO material **(8**). Care should be taken with screening antibodies in KO mice. In the H_3R -/- mice, the H_3R gene is disrupted by homologous recombination (US Patent no. 7151200). A 0.7 kb region covering part of the first intron and the 5' end of the second exon is replaced. Although the gene is rendered non-functional by this disruption with no detectable [^{3}H]-RAMH binding in KO animals (**16**; US Patent no. 7151200) there could possibly be some low level expression of non-functional H_3R protein remaining. This was a significant issue in some of the early H_3R KO mice. This is also unlikely to be the closely related H_4R; EAMPLHRGSK sequence is not found within the H_4R aa sequence and the anti-rH_{3C}/$hH_{3\ (445)}$ antibody does not detect recombinant H_4R expressed in HEK 293 cells. The high expression in the basal ganglia (Fig. 10), particularly in the substantia nigra, is consistent with recent double labelling confocal studies using these H_3 receptor antibodies. These have shown that the H_3 receptor is expressed on subpopulations of GABA-ergic neurons in the sustantia nigra and frontal cortex, and furthermore on subpopulations of histaminergic neurons in the tubermammilary nucleus, which suggests distinct control of output neurons to other brain regions by the H_3 receptor (**17**).

In conclusion, generating Human H_3R isoform specific antibodies requires a high level of knowledge and expertise. Thorough validation of the antibodies, as described herein, is essential before they can be used as reliable probes to investigate the physiological relevance of alternative splicing in the human H_3 receptor.

Dedication.

I would like to dedicate this chapter to the memory of my mum for her love and support over 5 decades.

References

1. Lovenberg TW, Roland BL, Wilson SJ, Jiang X, Pyati J, Huvar A, Jackson MR, Erlander MG. Cloning and functional expression of the human histamine H3 receptor. Mol Pharmacol. 1999 Jun;55(6):1101-7.

2. Arrang JM, Garbarg M, Schwartz JC. Auto-inhibition of brain histamine release mediated by a novel class (H3) of histamine receptor. Nature. 1983 Apr 28;302(5911):832-7.

3. Morisset S, Sasse A, Gbahou F, Héron A, Ligneau X, Tardivel-Lacombe J, Schwartz JC, Arrang JM. Biochem Biophys Res Commun. 2001 Jan 12;280(1):75-80.

4. Wiedemann P, Bönisch H, Oerters F, Brüss M. Structure of the human histamine H3 receptor gene (HRH3) and identification of naturally occurring variations. J Neural Transm (Vienna). 2002 Apr;109(4):443-53.

5. Cogé F, Guénin SP, Audinot V, Renouard-Try A, Beauverger P, Macia C, Ouvry C, Nagel N, Rique H, Boutin JA, Galizzi JP. Genomic organization and characterization of splice variants of the human histamine H3 receptor. Biochem J. 2001 Apr 15;355(Pt 2):279-88.

6. Harper EA, Shankley NP, Black JW. Evidence that histamine homologues discriminate between H3-receptors in guinea-pig cerebral cortex and ileum longitudinal muscle myenteric plexus. Br J Pharmacol. 1999 Oct;128(3):751-9

7. Chazot PL, Hann V, Wilson C, Lees G, Thompson CL. Immunological identification of the mammalian H3 histamine receptor in the mouse brain. Neuroreport. 2001 Feb 12;12(2):259-62

8. Cannon KE, Chazot PL, Hann V, Shenton F, Hough LB, Rice FL. Immunohistochemical localization of histamine H3 receptors in rodent skin, dorsal root ganglia, superior cervical ganglia, and spinal cord: potential antinociceptive targets. Pain. 2007 May;129(1-2):76-92. Epub 2006 Nov 28

9. Wellendorph P, Goodman MW, Burstein ES, Nash NR, Brann MR, Weiner DM. Molecular cloning and pharmacology of functionally distinct isoforms of the human histamine H(3) receptor. Neuropharmacology. 2002 Jun;42(7):929-40

10. Chazot PL, Pollard, S and Stephenson FA (1999) Immunoprecipitation of Receptors 257-285 In Vitro Neurochemical Techniques Editors: Boulton, Alan A., Baker, Glen B., Bateson, Alan N. (Eds.)

11. van Rijn RM, van Marle A, Chazot PL, Langemeijer E, Qin Y, Shenton FC, Lim HD, Zuiderveld OP, Sansuk K, Dy M, Smit MJ, Tensen CP, Bakker RA, Leurs R. Cloning and characterization of dominant negative splice variants of the human histamine H4 receptor. Biochem J. 2008 Aug 15;414(1):121-31

12. Shan L, Bossers K, Luchetti S, Balesar R, Lethbridge N, Chazot PL, Bao AM, Swaab DF. Alterations in the histaminergic system in the substantia nigra and striatum of Parkinson's patients: a postmortem study. Neurobiol Aging. 2012 Jul;33(7):1488.e1-13
13. Lethbridge N Histamine H_3 receptor heterogeniety in the Central nervous system in ageing and dementia. PhD thesis.

14. Shenton FC[1], Hann V, Chazot PL. Evidence for native and cloned H3 histamine receptor higher oligomers. Inflamm Res. 2005 Apr;54 Suppl 1:S48-9.

15. Bakker RA, Lozada AF, van Marle A, Shenton FC, Drutel G, Karlstedt K, Hoffmann M, Lintunen M, Yamamoto Y, van Rijn RM, Chazot PL, Panula P, Leurs R. Discovery of naturally occurring splice variants of the rat histamine H3 receptor that act as dominant-negative isoforms. Mol Pharmacol. 2006 Apr;69(4):1194-206

16. Toyota H, Dugovic C, Koehl M, Laposky AD, Weber C, Ngo K, Wu Y, Lee DH, Yanai K, Sakurai E, Watanabe T, Liu C, Chen J, Barbier AJ, Turek FW, Fung-Leung WP, Lovenberg TW. Behavioral characterization of mice lacking histamine H(3) receptors. Mol Pharmacol. 2002 Aug;62(2):389-97.

17. Giannoni P, Passani MB, Nosi D, Chazot PL, Shenton FC, Medhurst AD, Munari L, Blandina P. Heterogeneity of histaminergic neurons in the tuberomammillary nucleus of the rat. Eur J Neurosci. 2009 Jun;29(12):2363-74